Universidad Zaragoza
1542

Trabajo Fin de Máster

Encapsulamiento de Hemoglobina porcina como fuente de oxigenación en ingeniería de tejidos
Porcine Hemoglobin encapsulation as an oxygen carrier in tissue engineering

Autor

Jesús Javier Rivas Morales

Director

Pedro Miguel Baptista

Ponente

José Manuel García Aznar

ESCUELA DE INGENIERÍA Y ARQUITECTURA

2018

TRABAJOS DE FIN DE GRADO / FIN DE MÁSTER

Escuela de Ingeniería y Arquitectura Universidad Zaragoza

DECLARACIÓN DE AUTORÍA Y ORIGINALIDAD

(Este documento debe acompañar al Trabajo Fin de Grado (TFG)/Trabajo Fin de Máster (TFM) cuando sea depositado para su evaluación).

D./Dª. Jesús Javier Rivas Morales,

con nº de DNI 06278975K en aplicación de lo dispuesto en el art. 14 (Derechos de autor) del Acuerdo de 11 de septiembre de 2014, del Consejo de Gobierno, por el que se aprueba el Reglamento de los TFG y TFM de la Universidad de Zaragoza,

Declaro que el presente Trabajo de Fin de (Grado/Máster) Máster, (Título del Trabajo) Encapsulamiento de Hemoglobina porcina como fuente de oxigenación en ingeniería de tejidos,

es de mi autoría y es original, no habiéndose utilizado fuente sin ser citada debidamente.

Zaragoza, a 30 de Enero de 2018

Fdo: Jesús Javier Rivas Morales

If I have seen further it is by standing on the shoulders of Giants.
Isaac Newton

Agradecimientos

Al Centro de Investigaciones Biomédicas de Aragón, en especial a Pedro por poder realizara dicha tesis de máster en su laboratorio aprendiendo y sumando experiencias cada día. Y al Instituto de Nanociencias de Aragón, en especial, a Manuel Arruebo y Martin por su ayuda, dedicación y enseñanza durante este tiempo.

Por supuesto, quiero agradecer a mi familia todo el apoyo y confianza mostrada en mi. Mama, Papa, Carlos sin vuestra ayuda esto nunca seria posible.

A la universidad y en concreto a todos los profesores del máster, por admitirme y así poder continuar mi formación en un campo apasionante.

Tambien, a todos los compañeros del equipo: Joana, Iris, Pilar, Natalia, Manuel, Sara, Laurens y a Sofia también.

Y por último a todos los amigos que me llevo de esta gran etapa, "Los almorranos", Dani, Esther, Vanesa, Clara, Fran, Javi, Lorenzo y Jorge. Y bueno a los de "Comidas Uni" también, Yago, Sandra, Cris, etc.

¡Gracias a todos por contribuir de una manera u otra!

Resumen

Encapsulamiento de Hemoglobina porcina como fuente de oxigenación en ingeniería de tejidos

En la presente tesis de máster, se ha perseguido obtener un sistema de oxigenación para ingeniería de tejidos, mediante la encapsulación de hemoglobina porcina. Para ello diferentes métodos de síntesis de encapsulación han sido llevados a cabo caracterizando y analizando los resultados obtenidos.

En primer lugar se encapsulo hemoglobina siguiendo la técnica de doble emulsión-evaporación del disolvente siguiendo dos protocolos diferentes, se obtuvieron nano partículas de entre 40 y 200nm aproximadamente con resultados de eficiencia de encapsulamiento y carga del principio activo razonables. A continuación, se optimizo y se sintetizaron microcapsulas por medio de electrospray con un tamaño aproximado de una micra, pero su compleja recolección y los pobres valores de encapsulamiento y oxigenación hacen que no sea una opción demasiado viable. Finalmente se obtuvieron microparticulas por medio de microreactores, con tamaños desde 3 micras hasta 200 y con valores de oxigenacion no demasiado significantes.

El diseño de un protocolo adecuado para poder cuantificar la hemoglobina después de las diversas síntesis y así poder establecer las eficiencia de encapsulamiento y de carga también ha sido objeto de desarrollo en el presente trabajo, así como una sencilla prueba de oxigenación, donde se comprobó que las partículas creadas por doble emulsión, y en menor mediada, las creadas por microreactores conservan parte de la funcionalidad de la hemoglobina para oxigenar, haciéndolas candidatas para un mayor estudio y posible implementación en ingeniería de tejidos.

Abstract

Porcine Hemoglobin Encapsulation as an oxygenation carrier in tissue engineering

Creating artificial blood is one of the demands that the research world is trying to meet, both to cover the lack of blood transfusions and to improve the drawbacks they present, these are the reasons why oxygenation systems have been created based on the encapsulation of hemoglobin as artificial oxygenation systems known as Hemoglobin Based Oxygen Carriers (HBOC), these demands for oxygen systems also exist in other fields such as tissue engineering or organ transplantation maintenance, in this master thesis hemoglobin is encapsulated by different techniques to achieve a viable and functional oxygen transport system that can serve as a source of oxygenation in tissue engineering.

For this purpose, the double emulsion evaporation of the solvent encapsulation technique was used to obtain nanoparticles with sizes from approximately 40 to 200nm and acceptable encapsulation and loading values. Electrospray was another of the techniques used; after a process of optimization to find the optimal conditions, particles of approximately a micrometer quite homogeneous were obtained. However the difficulty in the collection and synthesis make its viability difficult as a system for large-scale production. Finally, microparticles were produced using microreactors, particles smaller than 42 micrometers, were the most frequent particles produced.

A simple oximetry test revealed that at least the nanoparticles generated by double emulsion evaporation of the solvent and via microreactors maintain some functionality for oxygenation, being a viable option for this purpose.

Contents

1 Introduction **1**

2 Materials and Methods **5**

2.1 Materials 5

2.2 Double emulsion synthesis 6

2.3 Electrospray 8

2.4 Sysnthesis via Microreactors 11

2.5 Encapsulation efficiency detection method 12

2.6 Oxygenation test 13

3 Results and Discussion **15**

3.1 Double Emulsion 15

3.1.1 Standard protocol 15

3.1.2 Scaled protocol 18

3.1.3 Synthesis using PLGA-PEG 20

3.2 Electrospray 21

3.3 Microreactors 24

3.4 Oxygenation test 25

4 Conclusion ad future works **27**

References **29**

List of Figures

1.1 Structure and configuration of hemoglobin [1] 1

1.2 Saturation oxygen curve [2] 2

2.1 Double emulsion-evaporation of the solvent diagram (W1/O/W2). 7

2.2 Electrospray Diagrams. 10

2.3 Hb spectophotometry 11

2.4 Microreactor system 12

2.5 Working in a nitrogen inert atmosphere 14

3.1 SEM images of the three samples using standard double emulsion technique 16

3.2 Size histograms of standard double emulsion synthesis 17

3.3 SEM images of the three samples using scaled double emulsion technique 18

3.4 Size histograms of Double Emulsion synthesis 19

3.5 Double emulsion synthesis using PLGA-PEG as polymer . . . 20

3.6 SEM images of different electrospraay tests. 21

3.7 Polidispersity histograms of different electrospray tests. 23

3.8 SEM images of electrospray synthesis at the conditios chosen . 24

3.9 Hb free microparticle generated by microreactors 24

3.10 Size histograms of microreactors synthesis 25

3.11 Microsphere via microreactors 25

List of Tables

2.1 List of chemical reagents used. 5

2.2 List of equipment used. 6

2.3 Optimization of electrospray encapsulation. 9

2.4 Solubility tests 13

3.1 Size average and standard deviation of double emulsion nanoparticles synthesis 16

3.2 Double emulsion size average and standard deviation 17

3.3 Scaled double emulsion size average and standard deviation . . 19

3.4 Double emulsion size average and standard deviation 20

3.5 Size average and standard deviation of electrospray tests. . . . 22

3.6 Electrospray size average and standard deviation 22

3.7 Oxygenation test results [O2 mg/ml] 26

Chapter 1

Introduction

Oxygen (O2) is an essential element for most living organisms to maintain their biological activities [3]. In humans and mammals, the main responsible protein for O2 delivery from the lungs to rest of tissues, is hemoglobin (Hb) [4]. The adult hemoglobin molecule (HbA) comprises four folded polypeptide chains (two *alpha* and two *beta*), each of which has a porphyrin heme group attached [5]. At the center of each of the four heme groups is an atom of iron in the ferrous (Fe^{2+}) state. These four iron atoms are the functional centers of the hemoglobin molecule because it is here that oxygen reversibly binds to form oxyhemoglobin, that is the protein state responsible for tissue oxygenation [6]. Hb is found inside red blood cells and its structure and configuration can be seen in Fig 1.1.

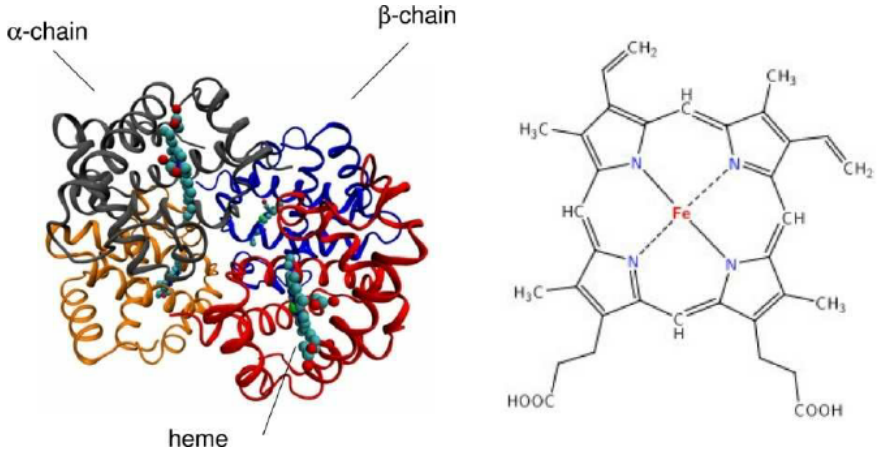


Figure 1.1: Structure and configuration of hemoglobin [1]

Oxygen binds to the ferrous haem group of Hb cooperatively, thus, the affinity that Hb has for oxygen is principally dependant on the local partial pressure of oxygen (pO2), and this affinity increases as a function of saturation following a sigmoidal curve with an approximate partial pressure of 26 mmHg for the 50% of saturation level. pH, temperature, or organic phosphates are also significant. [6, 7]. Fig. 1.2

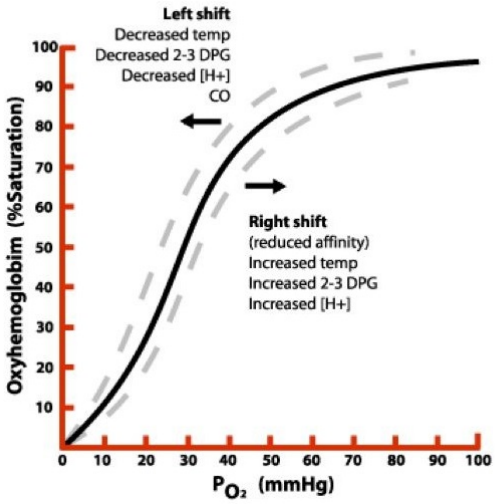


Figure 1.2: Saturation oxygen curve [2]

Thus, maintaining appropriate levels of Hb in the organism is important and indispensable for homeostasis. Hb deficiencies can cause various pathologies and difficulties in the oxygenation of the organism. However, there are some cases where Hb concentration drops to a lower level that may alter various biological activities, for example, severe injuries, car accidents, or surgical procedures produce Hb levels falls, in these cases, a blood transfusion is the only solution for re-establishing the appropriate levels [8]. Thus, there is a high demand for blood transfusion, but transfusions resources are limited and have some disadvantages, such as blood-type mismatching, special condition storage, infections caused by the presence of pathogens, and short stability [9–11]. Consequently it seems logical to find a solution, in order to solve these disadvantages.

A possible solution could be to use directly Hb as oxygen carrier but cell-free Hb cannot be used as a blood substitute due to its short circulation time,

toxicity, and high oncotic pressure [12]. To overcome these problems, attempts have been made to develop various types of oxygen deliver systems known as Hemoglobin-Based Oxygen Carriers (HBOCs). HBOCs are derived from outdated human or mammal blood as a source of Hb. [13,14]. Predominantly try to mimic the RBCs, introducing native or modified Hb inside of a capsule (nanocapsules or microcapsules) imitating the membrane of the cells and avoiding toxicity of cell-free Hb. [15–19]. Although HBOCs solve almost all of the problems of blood transfusions, as availability, lack of infections, long-term storage or universal compatibility [14,20], they also have some side effects, some of the most important will be discussed next.

When cell free Hb is released into the blood circulation, the tetramer structure of Hb spontaneously dissociates into two dimer configurations (*alpha* and *beta*) which leads to several side effects, whereas, inside the RBCs, the concentration of dimers is practically insignificant [16]. Dimers have a low molecular weight, approximately 32kDa, due to their size glomerular filtration occur causing nephrotoxicity [21]. The dimer haem iron is oxidized, more easily than in the tetramer, from Fe^{2+} to Fe^{3+} forming methemoglobin (MetHb) which is unable to bind oxygen. In addition, the formation of MetHb generates reactive oxygen and nitrogen species that can cause oxidative damage [7]. Whereas Hb inside of RBCs is protected against oxidation, moreover the presence of reductase system keeps MetHb levels very low. Another side effect reported in practically all of the studies is an increase of blood pressure due to scavenge of nitric oxide (NO), an important molecule that regulates the vascular tone. Dimers extravasate vascular walls and interact with NO, generating a vasoconstriction [7, 11, 14], thus increasing the molecular size of Hb in order to avoid this extravasation is one of the most used strategies to prevent hypertension. Keep Hb in its tetramer structure, not in dimers, avoids extravasation across vessels and kidneys, and provides a protective environment that limits the oxidation to MetHb and reduces the formation of potentially toxic oxidative products are the primordial aims of HBOCs design. Therefore, it seems logical that encapsulating Hb could be an optimal solution for solving these drawbacks.

During the present project, porcine Hb has been encapsulated by means of three procedures: via double emulsion evaporation of the solvent encapsulation technique, via electrospray and via microreactors. Each method of encapsulation will be described in depth in the next chapter. In all of them the polymer used was Poly(Lactic-Co-Glycolic Acid) (PLGA-COOH), which

was chosen due to its mechanical characteristics, biodegradation, and easy of use. In any case, it should be taken into account, that the main purpose of this master thesis will be to develop a viable and functional Hb encapsulation technique with a suitable particle size to try to avoid the absorption of particles by macrophages or other types of cells when this oxygenation system is used in tissue engineering. According to some studies, an optimal size, to avoid the particles internalization inside the cells would be between 70 and 200nm [2,16]. On the other hand, if we want to imitate the behavior and characteristics of red blood cells, micrometric capsules would be the most appropriate. Therefore, particles between 70 and 200nm or with micrometric size would be the most suitable for this application.

Chapter 2

Materials and Methods

2.1 Materials

Table 2.1 shows all reagents used during the present master thesis.

Reagent	Company
Acetic acid (99,98%)	Panreac
Acetone	Panreac
Dichloromethane (DCM) (99,98%)	Fisher Scientific
Dimethylformamide (DMF) (99,94%)	Fisher Scientific
Dimethyl sulfoxide (DMSO)	Sigma Aldrich
Ethyl acetate (99,5%)	Sigma-Aldrich
Hemoglobin porcine lyophilized powder	Sigma-Aldrich
Heptane (99%)	Acros Organics
Hydrochloric acid (37%)	Acros Organics
Iron standard for AAS	Fluka Analytical
Nitric acid (65%)	Panreac
Nitrile acetate	Panreac
PLGA, Poly(D,L-lactide-co-glycolide)	Sigma-Aldrich
Poly(ethylene glycol) methyl ether-block-poly(L-lactide-co-glycolide)	Sigma-Aldrich
Polyvinyl alcohol (PVA)	Sigma-Aldrich
Sodium collate	BioXtri
Tetrahydrofuran (THF) (99%)	Honeywell

Table 2.1: List of chemical reagents used.

In table 2.2 the equipment used during this research work is shown.

Equipment	Model	Company
Atmosbag glove	Z530204-1E	Sigma Aldrich
Atomic Emission Spectrometer	MP-AES 4100	Agilent
Centrifuge	MiniSpin	Eppendorf
Electrospinner	Electrospinner2,2-D	Yflow
Freeze-dryer	LyoQuest	Telstar
Multi-stirrer plate	MIX 6	2mag magnetic e motion
Oximeter	Orion Star A323	Thermo Scientific
Syringe Pump	PHD ULTRA	Harvard Apparatus
Sonicator	Sonifier 450	Branson Digital
Spectrophotometer	UV-Vis V-670	JASCO

Table 2.2: List of equipment used.

2.2 Double emulsion synthesis

The double emulsion evaporation of the solvent technique is used for the encapsulation of hydrophilic bioactive agents such as proteins, peptides, etc. It is a process consisting of the formation of a first emulsion (W1/O) between an organic phase containing the polymer and an aqueous phase containing the active principle. Subsequently, the emulsion formed, which contains the polymer and the active principle, is collected in an aqueous phase containing a surfactant that stabilizes the microparticles to form the second emulsion (W1/O/W2). Finally, the organic solvent evaporates, obtaining micro or nano particles loaded with our active principle [22] Fig. 2.1.

In our case Hb was encapsulated into the polymer PLGA-COOH following the protocol detailed below, which has been previously optimized for the production of polymeric particles in the group of Nanoporous Particles and Films (NFP) where part of this master thesis has been carried out.

- Dissolve 50mg of PLGA-COOH in 1ml of ethyl acetate.
- Dissolve 0.5mg Hb in 50ul of water Mili-Q.

- Mix both solutions in an ice bath and sonicate during 15 seconds with an amplitud of 30%.
- Add 2ml of 1% (w/v) sodium colate to Mili-Q water and sonicate in ice bath. Amplitude 30%, for 15 seconds.
- Add 10ml of 0.3% (w/v) sodium colate in Mili-Q water.
- Evaporate the solvent (ethyl acetate) by agitation. For 3 hours at 600rpm.
- Wash with Mili-Q water.
- Store in cold storage

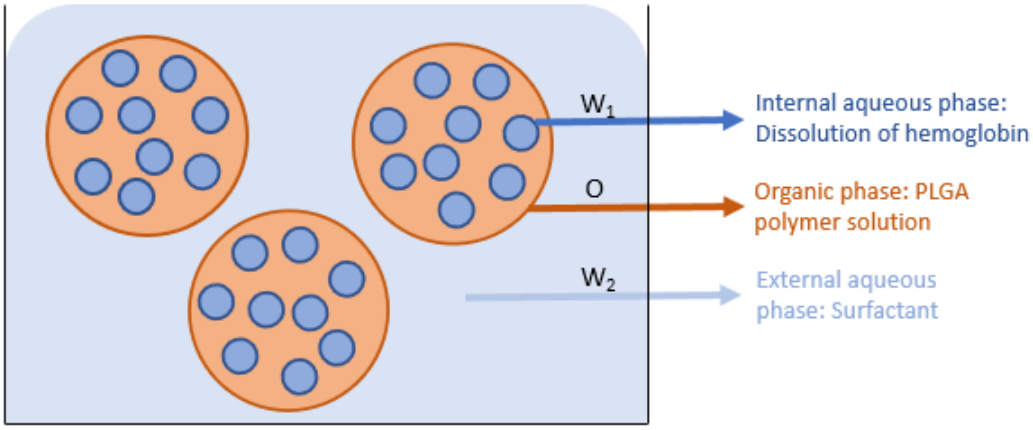


Figure 2.1: Double emulsion-evaporation of the solvent diagram (W1/O/W2).

In this protocol of double emulsion evaporation of the solvent some variables can be changed to get more drug encapsulation. The details of this scaled protocol are defined below.

- Dissolve 150mg of PLGA-COOH in 3ml of ethyl acetate.
- Dissolve 10mg Hb in 1ml of water Mili-Q.
- Mix both solutions sonicating in an ice bath with a 40% amplitude in the sonicator for 20 seconds.

- Add 8ml of 1% (w/v) sodium colate to Mili-Q water and sonicate in ice bath. Amplitude 40%, for 40 seconds.
- Add 12ml of 0.3% (w/v) sodium colate in Mili-Q water.
- Evaporate the solvent (ethyl acetate) by agitation. For 3 hours at 600rpm.
- Wash with Mili- Q water.
- Store in cold storage

Both protocols were used in Hb encapsulation. The results will be discussed in the following chapter.

2.3 Electrospray

Although the most commonly used and known term for this type of technique is electrospinning, in our case the appropriate term is electrospray, since electrospinning refers to the generation of nano or micro fibers, and for our application, we want to generate particles, therefore the appropriate word is electrospray. This technique consists of the synthesis of particles by means of electrostatic repulsion forces that exceed the weak forces of surface tension of the liquid that forms the suspensions of our polymer together with the active principle. After the electrospray process, dry particles of nanometric or micrometric size are obtained, charged with the drug, in our case Hb [23].

In this method of encapsulation, there are multiple factors that can cause the results obtained to vary drastically, some of these parameters are the following:

- **Flow rate:** knowing this parameter of the method allows prediction of the behavior that the material will have in determining particle size. In our synthesis the established flow rate was 0.5ml/h. A low flow to encourage solvent volatility.

- **Collector distance:** to control particles size and uniformity, the effective evaporation of the solvent must be taken into account, and this will depend on the collector distance and the evaporation rate of solvent. A distance of 30cm was chosen due to previous experience with this type of synthesis as well as to favour the evaporation of the solvent.

- **Electric potential:** this parameter is critical for the correct encapsulation procedure, because it affects the size and form of the droplets. A potential of sufficient electric charge can overcome the surface tension of polymer solution to produce micro or nano particles.

- **Polymer/Hb relation:** the relationship between polymer and protein will affect several factors such as: viscosity, electrical conductivity or surface tension, very important parameters in particle synthesis.

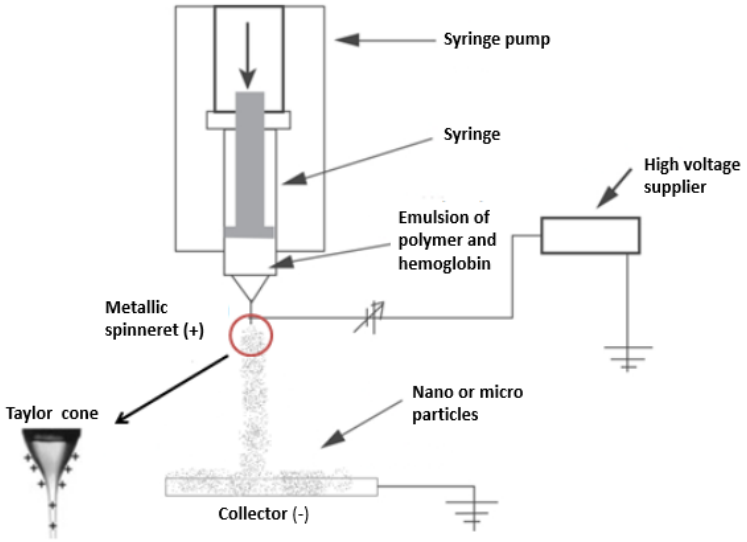
A theoretical diagram of this technique, as well as the current system used during the present thesis are shown in Fig. 2.2.

In order to obtain optimal encapsulation results by electrospray, different electric potential and polymer/Hb relation values were tested. The different tests carried out are summarized in Table 2.3. It should be taken into account that the voltage values listed in this table correspond to the negative voltage (collector) and the positive voltage (high voltage supplier).

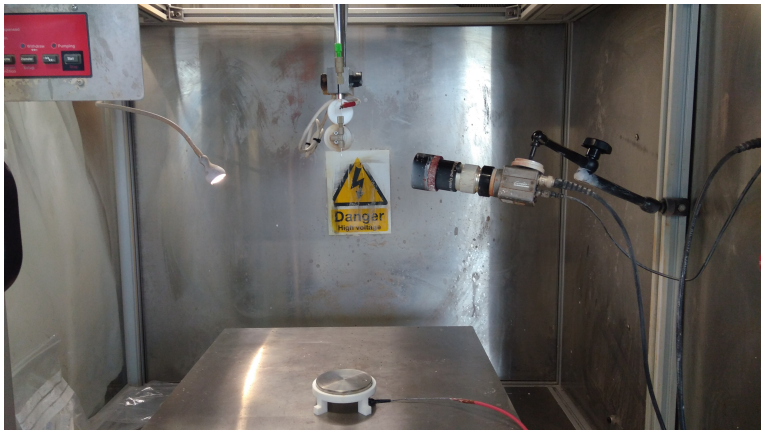
Samples	Polymer/Hb (wt/wt) relation	Voltage [Kv]
Sample 1	100:1(1%)	-3/+9,5
Sample 2	100:1(1%)	-3/+13
Sample 3	100:1,25(1,25%)	-3/+13
Sample 4	100:1,5(1,5%)	-3/+13
Sample 5	50:1(2%)	-3/+13

Table 2.3: Optimization of electrospray encapsulation.

The polymer used (PLGA-COOH) was dissolved in dimethylformamide (DMF) (with a 10% w/v ratio) while Hb is found as an aqueous solution. Therefore, in order to have both reagents in the same solution, both solutions were added and by means of mechanical agitation at 750rpm overnight in the cold-storage room, a stable emulsion is formed, which will be the product used in the electrospray. To confirm that with this emulsion, Hb has not been



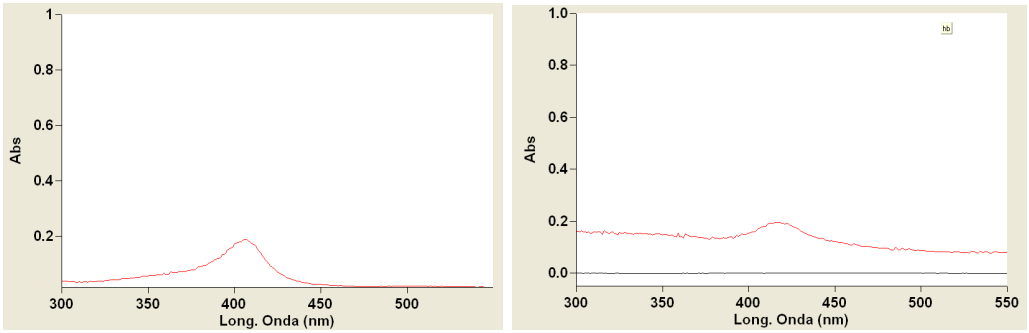
(a) Electrospray theoretical diagram [24]



(b) Real electrospray system used

Figure 2.2: Electrospray Diagrams.

denatured or lost its properties we will test this emulsion in the spectrophotometer, as can be seen in Fig. 2.3, the solution of Hb and polymer has a peak similar (Fig. 2.3b) to Hb in the aqueous solution (Fig. 2.3a).



(a) Hb solution (b) Emulsion of Hb with dissolved polymer

Figure 2.3: Hb spectophotometry

2.4 Sysnthesis via Microreactors

In this case by means of this method particles of micrometric size will be generated. The microreactor consists of two coaxial capillaries, the internal capillary is made of PEEK and has a dimension of 150 micrometers. The external capillary is made of PTFE and has a dimension of 0.03 inches (approximately 760 micrometers) for each capillary we will have a solution. On the one hand, the organic solution with polymer and Hb dissolved in the organic solvent, in our case dichloromethane (DCM)(internal capillary), and on the other hand the aqueous phase formed by water with Polyvinyl alcohol (PVA)(1%w/v)(external capillary), both flows will be made to pass through a microreactor generating small particles that will be collected and after the evaporation of the solvent, the desired particles will be obtained.

In the Fig.2.4 it is possible to observe the system assembly with the two flows and the microreactor.

First tests were performed without Hb to establish the appropiate encapsulation system. Thus, the first tests were carried out with the following characteristics. For the organic phase, 10 mg of polymer (PLGA-COOH) were added per milliliter of solvent (DCM) with a flow rate of 0.5ml/h. While

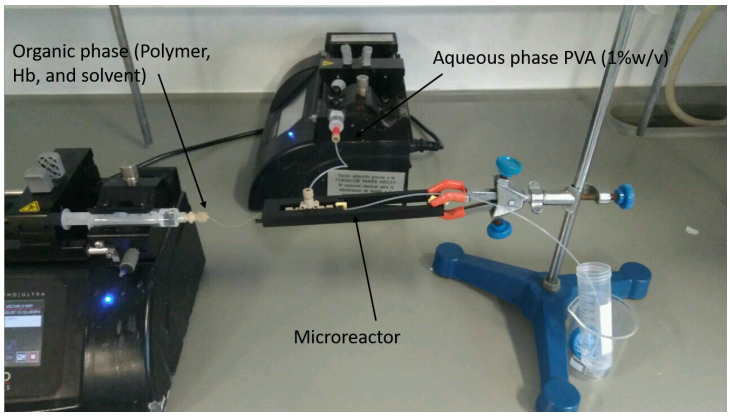


Figure 2.4: Microreactor system

in the aqueous phase PVA (1% w/v) was added with a flow rate of 5ml/h. Obtaining microparticles, whose results will be discussed in the next chapter.

Once the system was stabilized to form microparticles, Hb was added to the organic phase. By agitation of the solvent, polymer and Hb at a temperature of 4ºC for 12h a stable emulsion (Oil/Water) was obtained for the synthesis via microreactors. This is the same procedure used for electrospray synthesis. A 5% of Hb was added in relation to the polymer, that is, 0.5mg of Hb per milliliter of solvent.

2.5 Encapsulation efficiency detection method

When a drug, protein, etc. is encapsulated, one of the most important factors that must be known is encapsulation efficiency (EE%), which refers to the amount of active principle that has been encapsulated with respect to the initial amount of the principle before synthesis. Normally in the NFP group, where part of this master thesis has been carried out, the following steps are followed to quantify the EE%: once the nanoparticles have been obtained, an organic solvent is added to break down the particles and dissolve the polymer and the drug, then some compound is added for changing the polarity of the solution, such as methanol, making the polymer precipitate to be able to eliminate it, finally the amount of the active principle is quantified by spectrophotometry. In our case, this method of analysis cannot be used, because by adding any organic solvent we denature the protein and the signal in spectrophotometry is not valid for its measurement.

Therefore, in order to measure the EE%, a new method of detection had to be implemented, for this purpose, one of the essential characteristics of Hb will be used, and it is the presence of four heme groups with one Fe group each one of them. Thus, if we can measure the presence of these Fe groups, we can know the amount of Hb present in the solution. To do this we followed the next process. Once the nanoparticles have been synthesized, an organic solvent is added that dissolves the polymer but does not dissolve our protein, in this case we do not care if the solvent denatured Hb, since we will measure the amount of Fe not the spectrophotometry signal. To find the appropriate organic solvent, a battery of solubility tests were performed among some of the most used organic solvents. (Table 2.4).

Reagent	Polymer solubility	Hb solubility
Acetic acid	Yes	Yes
Acetone	Yes	Partially
Dichloromethane (DCM)	Yes	Partially
Dimethylformamide (DMF)	Yes	No
Dimethyl sulfoxide (DMSO)	Yes	Yes
Ethyl acetate	Yes	Partially
Heptane	No	Partially
Nitrile acetate	Yes	Partially
Tetrahydrofuran (THF)	Yes	Partially

Table 2.4: Solubility tests

Finally DMF was chosen, because this solvent dissolves the polymer but not Hb, so by means of centrifugation, Hb can be collected. Once we have the Hb pellet, 15% of acid (regious water or acetic acid) will be added to make the iron free in the solution and can be measured by microwave plasma atomic emission spectroscopy. Once the concentration of iron is known, the amount of Hb can be calculated.

2.6 Oxygenation test

Perhaps the most important test in order to validate this thesis is to verify that the encapsulated Hb is functional and able to bind oxygen, since this is the main purpose of this study. A simple test was performed to validate

whether the particles can actually oxygenate. The test was carried out following these steps: first argon was introduced into water samples to displace the oxygen content and get a sample practically deoxygenated, later pure Hb, particles designed through different types of synthesis and water as a control, was added to desoxygenated water samples and with the help of an oximeter the amount of oxygen was measured. In any case, all of the measures were carried out in an inert nitrogen atmosphere in order to the oxygen in the air not affect the results obtained, for this a closed bag that only contained nitrogen was used (Fig. 2.5).

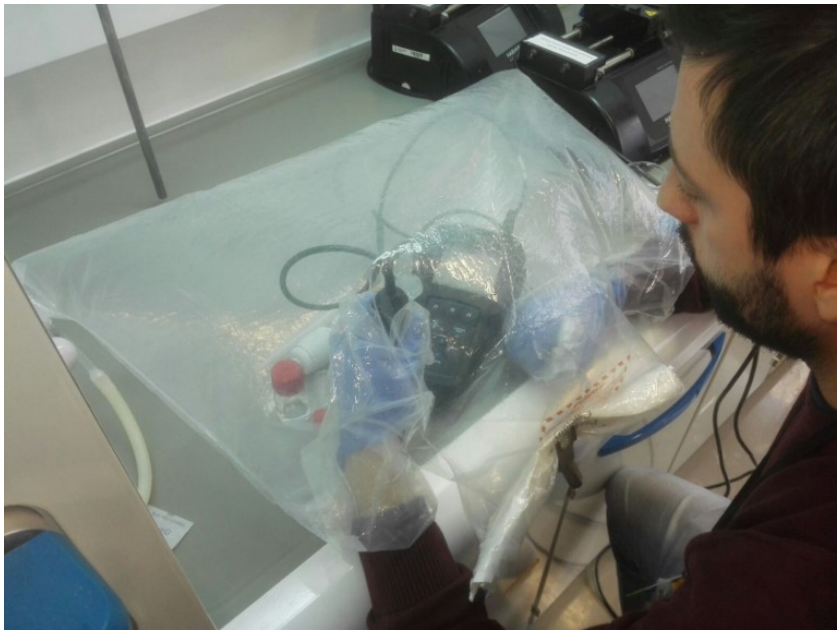


Figure 2.5: Working in a nitrogen inert atmosphere

Chapter 3

Results and Discussion

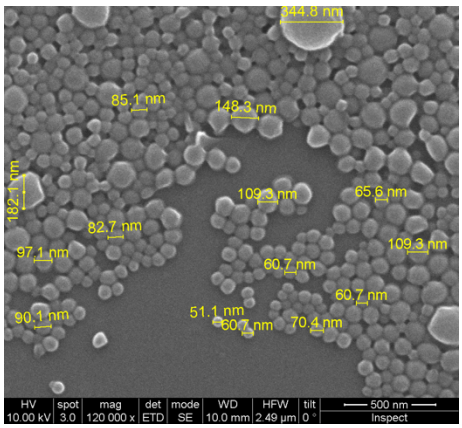
3.1 Double Emulsion

3.1.1 Standard protocol

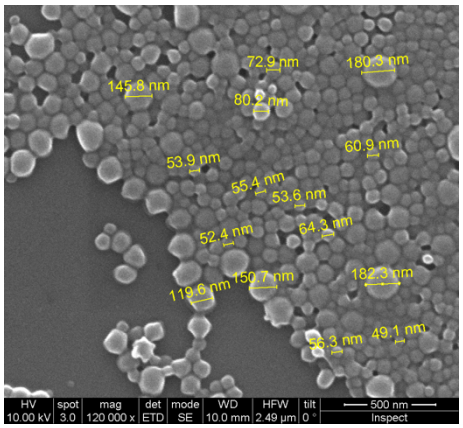
The following results are related to the double emulsion evaporation of the solvent encapsulation technique following standard protocol, results of scaled synthesis will be discussed in the next section, both procedures were described previously. Size and shape will be studied using images taken by scanning electron microscopy (SEM). It will also study its polydispersity representing its sizes histogram. Finally, the EE% and drug loading will be analyzed following the technique described in the previous chapter.

In the Fig. 3.1 it is possible to appreciate the nanoparticles created by three different replicas. It can be observed, at a glance, that the particle sizes range are from approximately 30nm to 150nm, although there are some exceptions. This synthesis shows a greater than expected polydispersity, this can be caused by wear or malfunction of the homogenizer, which is a determinant factor in this type of synthesis.
It can also be seen how the nanoparticles have approximately spherical shape.

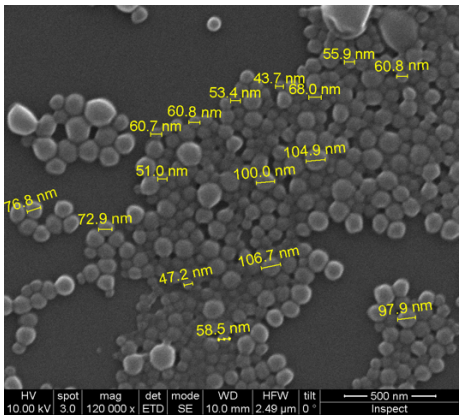
The average particle size and its standard deviation are shown in Table 3.1. An average size of approximately 100nm was obtained with a standard deviation of about 35.



(a) Double emulsion, SEM image. Sample 1



(b) Double emulsion, SEM image. Sample 2



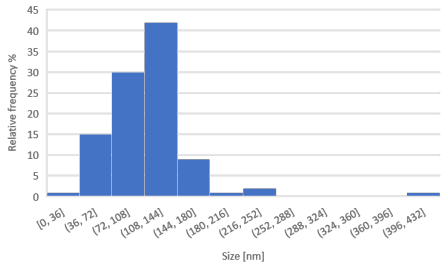
(c) Double emulsion, SEM image. Sample 3

Figure 3.1: SEM images of the three samples using standard double emulsion technique

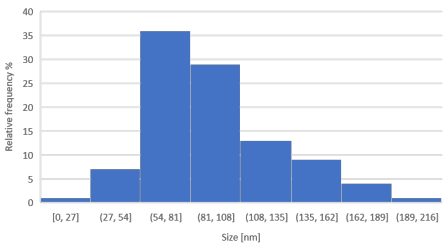
Samples	Average size[nm]	Standard Deviation
Sample 1	104,94	44,41
Sample 2	96,3	32,67
Sample 3	97,34	32,25

Table 3.1: Size average and standard deviation of double emulsion nanoparticles synthesis

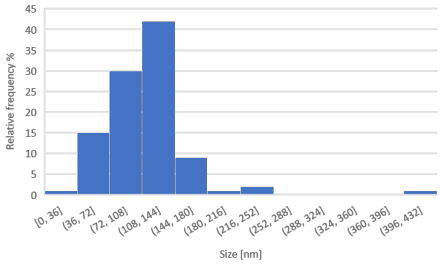
By observing size hisstograms (Fig. 3.2), the polydispersity of nanoparticles is confirmed, having a wide range of sizes although the size range between approximately 60 and 120nm is the most frequent.



(a) Size histogram. Sample 1



(b) Size histogram. Sample 2



(c) Size histogram. Sample 3

Figure 3.2: Size histograms of standard double emulsion synthesis

After performing numerous Hb measurements at known concentration values, an error of approximately 30% in the measure was detected, this loss of measurement can be due to the impurities of Hb, or the incomplete separation of Fe groups from Hb. Therefore, to the values obtained from EE%, 30% was added to compensate this error in the measurement (EE% correction). This procedure was just used to obtain an approximation of the efficiency and load values. Further studies to eliminate this deficiency must be carried out.

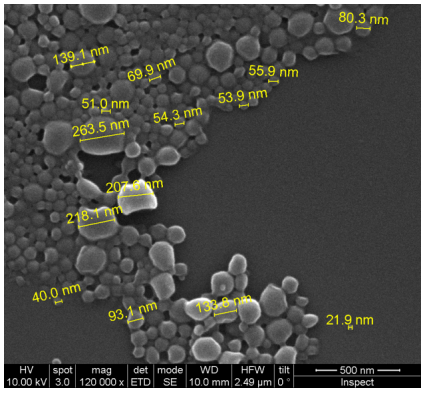
Samples	EE%	EE% (correction)	Drug Loading((wt%))
Sample 1	22,34	32,39	5,71
Sample 2	27,43	39,77	4,63
Sample 3	29,72	43,09	5,13
Average	26,5	38,42	5,16
Satandard deviation	3,78	5,48	0,54

Table 3.2: Double emulsion size average and standard deviation

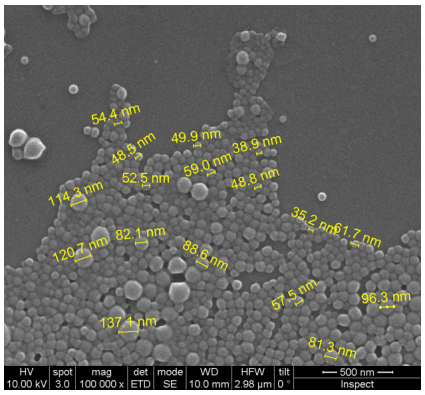
On the other hand, drug loading is a parameter that represents the amount of total entrapped drug, in our case Hb, divided by the total nanoparticle weight. It is used to know the total amount of active principle delivered when a known amount of nanoparticles is released. In this case the values of loading are acceptable, since it is difficult to get values higher than 10% [25].

3.1.2 Scaled protocol

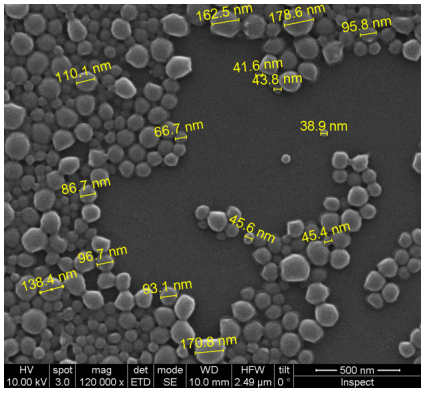
Below are the results for the synthesis of nanoparticles via double emulsion evaporation of the solvent but following the scaled protocol, which is usually used to try to introduce more active principle into the particles, effect that we will check later. As before, the images collected through SEM (Fig. 3.3) will be used to know the size and shape of the nanoparticles obtained.



(a) Double Emuslsion. Sample 1



(b) Double Emuslsion. Sample 2



(c) Double Emuslsion. Sample 3

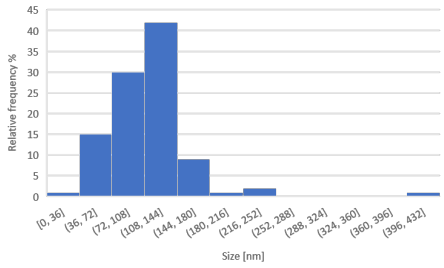
Figure 3.3: SEM images of the three samples using scaled double emulsion technique

In this case it seems that the particle size has been reduced a little, a fact that is confirmed by the results listed in the Table 3.3 where it can be observed how the average size is 60nm with a standard deviation of approximately 25.

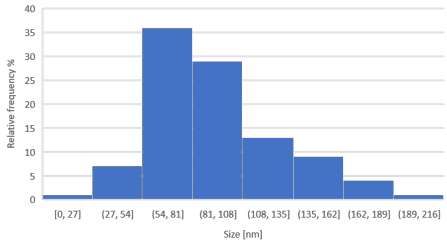
Samples	Average size[nm]	Standard Deviation
Sample 1	59,70	24,08
Sample 2	58,14	25,93
Sample 3	62,964	28,91

Table 3.3: Scaled double emulsion size average and standard deviation

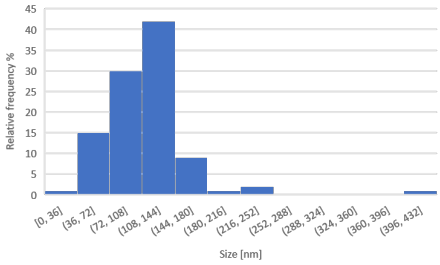
Histograms show polydispersity, being the size range of 40 to 80nm is the most frequent.



(a) Double Emuslsion. Sample 1



(b) Double Emuslsion. Sample 2



(c) Double Emuslsion. Sample 3

Figure 3.4: Size histograms of Double Emulsion synthesis

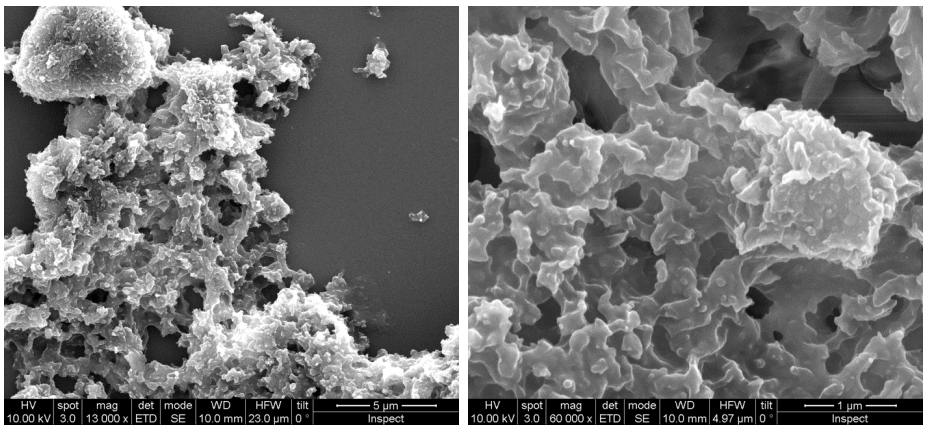
Finally, we analyzed the results of EE% and drug loading. As previously mentioned, this scaled technique is often used to trap more compound inside the particles. If we observe the Table 3.4 it can be reported that with this protocol the EE% and drug loading has increased.

Samples	EE%	EE% (correction)	Drug loading (wt%)
Sample 1	40,01	57,99	7,46
Sample 2	36,20	52,51	6,76
Sample 3	34,35	48,76	7,01
Average	36,88	53,08	7,07
Satandard deviation	2,91	4,65	0,35

Table 3.4: Double emulsion size average and standard deviation

3.1.3 Synthesis using PLGA-PEG

In order to obtain a greater circulation time and biocompatibility, it was attempted to synthesize nanoparticles via double emulsion evaporation of the solvent but using PLGA-PEG as polymer, instead of PLGA-COOH, a replication with each protocol was carried out, however no particles were obtained. In the Fig. 3.5 it can be appreciated how neither with the standard protocol nor with the scaled one the objective was achieved. These negative results may be due to the fact that procedures used in this synthesis were optimized for PLGA, not PLGA-PEG, therefore no particles formation or encapsulation has taken place, if we want to obtain positive results we should carry out an optimization, checking different solvents, surfactants, etc.



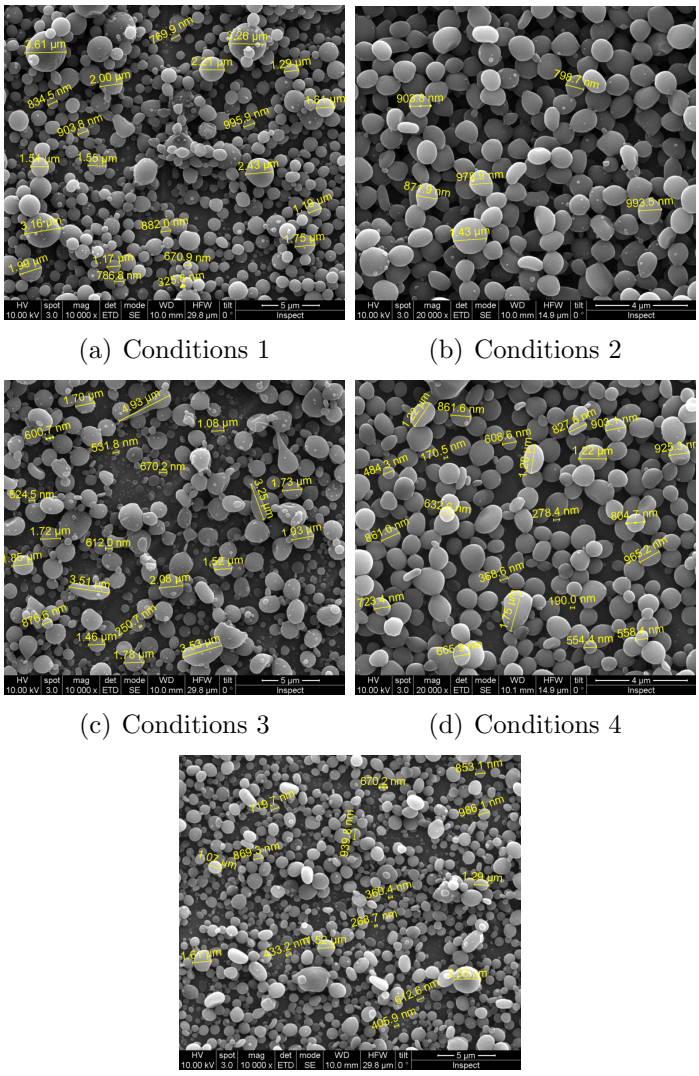
(a) Standard double emulsion using PLGA-PEG as polymer

(b) Scaled double emulsion using PLGA-PEG as polymer

Figure 3.5: Double emulsion synthesis using PLGA-PEG as polymer

3.2 Electrospray

The results of the images taken by SEM from the different tests can be seen in Fig. 3.6.



(a) Conditions 1 (b) Conditions 2

(c) Conditions 3 (d) Conditions 4

(e) Conditions 5

Figure 3.6: SEM images of different electrospraay tests.

As can be seen from the different conditions analyzed, images with conditions 2 and 4 appear to be the most viable options because with these conditions,

particles obtained present more stable and homogeneous size and the lowest standard deviation. Analyzing the results in detail (Table 3.5) and making the size histograms (Fig. 3.7) to check the polydispersity, it seems that the best option is condition 4, because it presents less polydispersity and less standard deviation. Thus these were the conditions chosen for the encapsulation procedure.

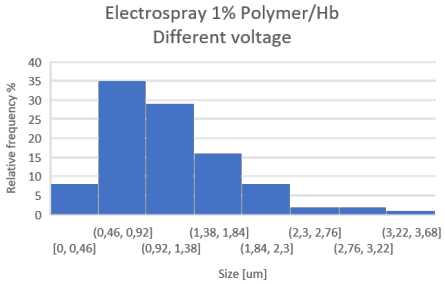
COnditions	Size average[um]	Standard deviation
Conditions 1	1,15	0,57
Conditions 2	0,85	0,31
Conditions 3	0,97	0,53
Conditions 4	0,78	0,27
Conditions 5	0,84	0,35

Table 3.5: Size average and standard deviation of electrospray tests.

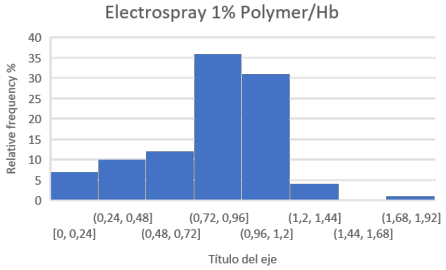
Samples	EE%	EE% (correction)	Drug loading (wt%)
Sample 1	12,51	18,1	3,23
Sample 2	9,62	13,93	2,96
Sample 3	8,45	12,28	2,84
Average	10,18	14,73	3,01
Standard deviation	2,09	3,05	0,2

Table 3.6: Electrospray size average and standard deviation

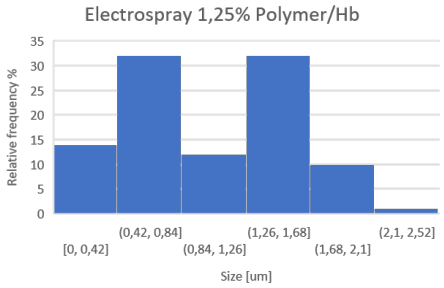
As can be seen in the SEM images, the synthesis of nanoparticles via slectrospray at the chosen conditions presents a good size and shape homogeneity (Fig. 3.8). However, the results obtained from EE% and drug loading are poor (Table 3.6), this can be explained by the particles collection method, since particles are collected in a metal paper surface. Later this collector must be scraped to obtain the particles in aqueous solution and be able to do the washing and analysis process. Throughout this process a lot of particles are lost. Moreover, during the synthesis part of nanoparticles is not deposited in the collector. Therefore, EE% and drug loading values should be taken carrefully, and knowing that factors previously exposed affect the final results.



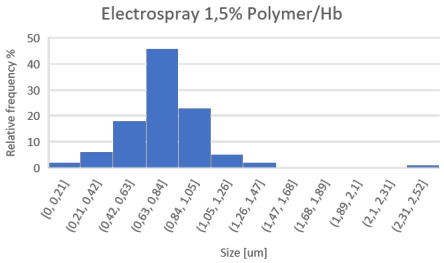
(a) Size histogram. Conditions 1



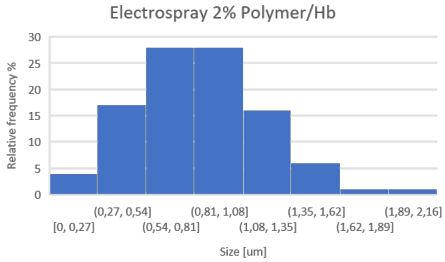
(b) Size histogram. Conditions 2



(c) Size histogram. Conditions 3

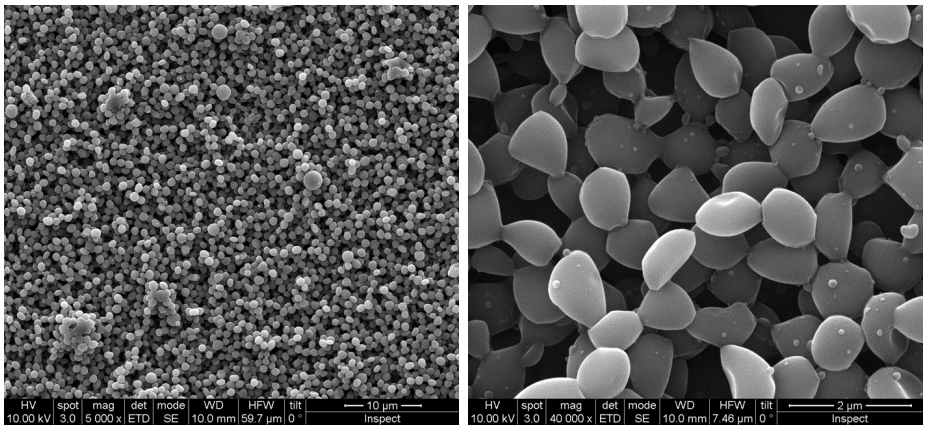


(d) Size histogram. Conditions 4



(e) Size histogram. Conditions 5

Figure 3.7: Polidispersity histograms of different electrospray tests.



(a) General electrospray SEM image (b) SEM image of electrospray synthesis in detail

Figure 3.8: SEM images of electrospray synthesis at the conditios chosen

3.3 Microreactors

As can be appreciated in the images taken by microscope (Fig 3.9), Hb free microparticles were generated with a large size range, from several micrometers to hundreds. Histogram was performed to check its polydispersity, being microparticles smaller than 42 micrometers the most abundant in the sample (Fig. 3.10).

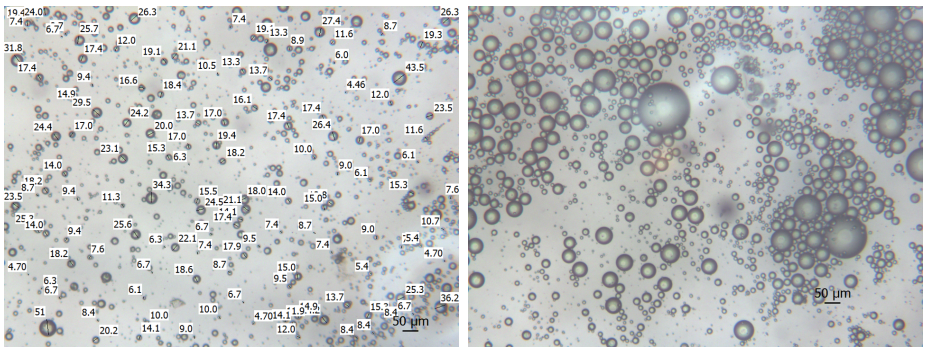


Figure 3.9: Hb free microparticle generated by microreactors

While the results obtained with the charged particles are shown below. The results in terms of size and shape were quite similar.

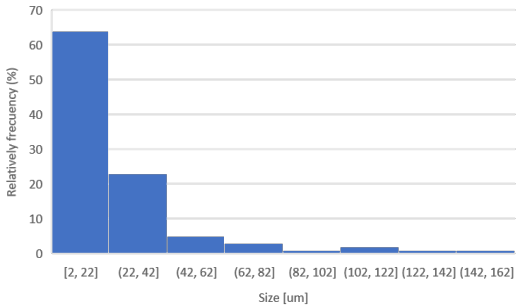
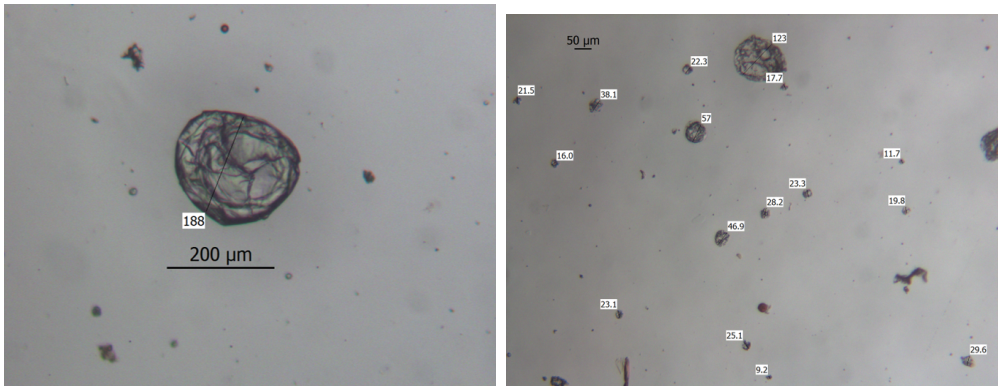


Figure 3.10: Size histograms of microreactors synthesis

In the Fig. 3.11 it is possible to observe the microcapsules loading with Hb created by microreactors, no significant differences with respect to the values obtained with free Hb microparticles are observed. In terms of shape, rough spheres are appreciated, characteristic of microparticles.



(a) Microsphere loading with Hb in detail (b) Microparticles loading with Hb. General overview

Figure 3.11: Microsphere via microreactors

3.4 Oxygenation test

The data for the oxygenation test are listed below. The main point of this simple test was to prove that at least some of the Hb functionality is preserved after encapsulation processes. According to the information collected, the standard double emulsion synthesis seems to show a significant oxygenation with respect to the sample with water used as a control, while in the results

of electrospray synthesis there are hardly any significant differences with the control sample, particles obtained via microreactor seems to preserve some functionality, although deeper studies should be carried out. Finally all measurements were taken at a homogeneous temperature of approximately 21°C.

Samples	Hb pure	Double Emulsion	Electrospray	Microreactors	H2O
Sample 1	2,13	1,65	1,32	1,26	1,18
Sample 2	2,34	1,56	1,14	1,34	1,21
Sample 3	2,37	1,77	1,11	1,38	1,09
Average	2,28	1,67	1,19	1,33	1,16
SD	0,13	0,09	0,11	0,06	0,06

Table 3.7: Oxygenation test results [O2 mg/ml]

However, these results should be taken with some caution, as it is only a first evidence that our particles can oxygenate the samples of deoxygenated water, much deeper and more complex tests must be carried out to implement this system in a real environment.

Chapter 4

Conclusion ad future works

In this master's thesis different encapsulation techniques have been used to find a functional and useful method of oxygenation using Hb as an active principle.

On the one hand, the synthesis of nanoparticles by means of double emulsion-evaporation of the solvent presents reasonable values for both encapsulation and drug loading, especially following the scaled method. In addition, by means of the oxygenation test, it has been proved that some of the Hb present remains functional. Its nanometric size (feasible to be trapped by certain cells such as macrophages) and its polydispersity, perhaps are its major disadvantages. However, its easy synthesis, and its reasonable results make it a viable option.

On the other hand, the synthesis optimized by electrospray presents some size and polydispersity results suitable for this application, although its difficulty in the collection and analysis of EE, besides the poor results obtained in the oxygenation test, do not make it a very applicable option.

Finally, the synthesis of microparticles via microreactors seems to be a promising option both for their micrometric size (avoiding the inclusion of particles in the cells) and for their continuous production avoiding possible discrepancies in batch production. Although it should be taken into account that its EE% and drug loading are not excessively good. In addition, the oxygenation values were worse than the ones obtained by double emulsion evapora-

tion synthesis. More tests should be carried out to optimize the process and achieve better results.

Regarding the possible future works that could be carried out, continue working on the optimization of the synthesis of microparticles via microreactors would be an excellent option.

Another future work would be to carry out deeper oxygenation tests in order to know, for example, the saturation pressure of Hb. Testing the micro or nano particles in cells to know the results in a real environment could be the definitive evidence to implement these oxygenation systems.

Finally, another interesting work would be to introduce other molecules into the particles such as the enzyme cytochrome b5 reductase, which is capable of converting methemoglobin (an oxidized state of hemoglobin that is unable to carry and deliver oxygen) into functional Hb again.

References

[1] Tao Wu, Xiaodong Wang, Barry Cohen, and Hongya Ge. Molecular modeling of normal and sickle hemoglobins. 8:237–244, 01 2010.

[2] Chris Nickson. Oxygen-haemoglobin dissociation curve.

[3] Kazuaki Taguchi, Keishi Yamasaki, Toru Maruyama, and Masaki Otagiri. Functional Biomaterials Comparison of the Pharmacokinetic Properties of Hemoglobin-Based Oxygen Carriers. 2017.

[4] Feng Xu, Yuan Yuan, Xiaoqian Shan, Changsheng Liu, Xinyi Tao, Yan Sheng, and Huanjun Zhou. Long-circulation of hemoglobin-loaded polymeric nanoparticles as oxygen carriers with modulated surface charges. *International Journal of Pharmaceutics*, 377(1-2):199–206, 2009.

[5] HM Ranney and V. Sharma. Structure and function of hemoglobin. *William's Hematology*, 2000.

[6] Chris Higgins. Methehemoglobin. *Blood gases and/or acid-base*, 2006.

[7] Andrea Mozzarelli, Luca Ronda, Serena Faggiano, Stefano Bettati, and Stefano Bruno. Haemoglobin-based oxygen carriers: Research and reality towards an alternative to blood transfusions. *Blood Transfusion*, 8(SUPPL. 3):59–68, 2010.

[8] Erhard Seifried and Markus M. Mueller. The present and future of transfusion medicine. *Blood Transfusion*, 9(4):371–376, 2011.

[9] Shahid Rameez, Nicole Guzman, Uddyalok Banerjee, Jorge Fontes, Michael E. Paulaitis, Andre F. Palmer, Rakesh P. Patel, and Jaideep Honavar. Encapsulation of hemoglobin inside liposomes surface conjugated with poly(ethylene glycol) attenuates their reactions with gaseous ligands and regulates nitric oxide dependent vasodilation. *Biotechnology Progress*, 28(3):636–645, 2012.

[10] Wenliang Li, Wenjing Zhang, Xiuyun Yang, Zhigang Xie, and Xiabin Jing. Biodegradable polymersomes from four-arm PEG-b-PDLLA for encapsulating hemoglobin. *Journal of Applied Polymer Science*, 131(12):1–5, 2014.

[11] Toby A., M.D. Silverman, Richard B., and M.D Weiskopf. Hemoglobin-based Oxygen Carriers Session I : Setting the Stage. *Anesthesiology*, (5):946–963, 2009.

[12] T. M.S. Chang. Nanobiotechnology for Hemoglobin-based Blood Substitutes. *Critical Care Clinics*, 25(2):373–382, 2009.

[13] Li Wang, Fang Liu, Kunping Yan, Wencan Pan, Lijuan Xu, Huifang Liu, Chengbin Yan, Chao Chen, and Hongli Zhu. Effects of resuscitation with polymerized porcine hemoglobin (pPolyHb) on hemodynamic stability and oxygen delivery in a rat model of hemorrhagic shock. *Artificial Cells, Nanomedicine, and Biotechnology*, 45(1):51–57, 2017.

[14] Abdu I. Alayash. Blood substitutes: Why haven't we been more successful?, 2014.

[15] Quan Wang, Ruirui Zhang, Mingzi Lu, Guoxing You, Ying Wang, Gan Chen, Caiyan Zhao, Zhen Wang, Xiang Song, Yan Wu, Lian Zhao, and Hong Zhou. Bioinspired Polydopamine-Coated Hemoglobin as Potential Oxygen Carrier with Antioxidant Properties. *Biomacromolecules*, 18(4):1333–1341, 2017.

[16] Mingxian Liu, Lihua Gan, Liuhua Chen, Dazhang Zhu, Zijie Xu, Zhixian Hao, and Longwu Chen. A novel liposome-encapsulated hemoglobin/silica nanoparticle as an oxygen carrier. *International Journal of Pharmaceutics*, 427:354–357, 2012.

[17] Wei Zhang, Kunping Yan, Penggao Dai, Jingjing Tian, Hongli Zhu, and Chao Chen. A Novel Hemoglobin-Based Oxygen Carrier, Polymerized Porcine Hemoglobin, Inhibits H 2O 2-Induced Cytotoxicity of Endothelial Cells. *Artificial Organs*, 36(2):151–160, 2012.

[18] Mingzi Lu, Caiyan Zhao, Quan Wang, Guoxing You, Ying Wang, Hongzhang Deng, Gan Chen, Sha Xia, Jingxiang Zhao, Bo Wang, Xianlei Li, Leihou Shao, Yan Wu, Lian Zhao, and Hong Zhou. Preparation, characterization and in vivo investigation of blood-compatible hemoglobin-loaded nanoparticles as oxygen carriers. *Colloids and Surfaces B: Biointerfaces*, 139:171–179, 2016.

[19] Ying Wang, Linli Wang, Weili Yu, Dawei Gao, Guoxing You, Penglong Li, Shan Zhang, Jun Zhang, Tao Hu, Lian Zhao, and Hong Zhou. A PEGylated bovine hemoglobin as a potent hemoglobin-based oxygen carrier. *Biotechnology Progress*, 33(1):252–260, 2017.

[20] Mary Njoku, Deidre St Peter, and Colin F Mackenzie. Haemoglobin-based oxygen carriers: indications and future applications. *British Journal Of Hospital Medicine (London, England: 2005)*, 76(2):78–83, 2015.

[21] Wei Gao, Baoyong Sha, Wei Zou, Xuan Liang, Xiangzhong Meng, Hao Xu, Jun Tang, Daocheng Wu, Lixian Xu, and Hui Zhang. Cationic amylose-encapsulated bovine hemoglobin as a nanosized oxygen carrier. *Biomaterials*, 32(35):9425 – 9433, 2011.

[22] M N Singh, K S Y Hemant, M Ram, and H G Shivakumar. Microencapsulation: A promising technique for controlled drug delivery. *Research in pharmaceutical sciences*, 5(2):65–77, jul 2010.

[23] Nandana Bhardwaj and Subhas C. Kundu. Electrospinning: A fascinating fiber fabrication technique. *Biotechnology Advances*, 28(3):325–347, may 2010.

[24] Nuno M Neves, Rui Campos, Adriano Pedro, José Cunha, Francisco Macedo, and Rui L Reis. Patterning of polymer nanofiber meshes by electrospinning for biomedical applications. *International journal of nanomedicine*, 2(3):433–48, 2007.

[25] Wenchao Bao, Jiaxiang Zhou, Jiufu Luo, and Dengxi Wu. PLGA microspheres with high drug loading and high encapsulation efficiency prepared by a novel solvent evaporation technique. *Journal of Microencapsulation*, 23(5):471–479, jan 2006.